Human Embryonic Stem Cells and Type I Diabetes: How Far to the Clinic?

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Introduction

Diabetes affects an estimated 16 million people in the United States[1] and more than 150 million people worldwide—and the prevalence of this disease is expected to double in the next 25 years.[2] Diabetes is the sixth leading cause of death in the United States,[1] the leading cause of blindness and amputation in developed countries, and the leading cause of renal failure and kidney transplant worldwide.[2] Compared with the nondiabetic population, people with diabetes are from two to four times more likely to have heart disease and are from two to four times more likely to have a stroke.[1] The total direct and indirect economic cost of diabetes in the United States in 2002 was estimated to be more than $132 billion.[1] Current therapeutics consist of insulin for patients with type I diabetes; for patients with type 2 diabetes, medications are used to stimulate insulin production (sulfonylurea and meglitinide agents), to enhance insulin sensitivity (bigaunide/metformin and glitazone), to decrease glucose absorption (glucosidase inhibitors), or to prevent overproduction of glucose by the liver (metformin).

Current Therapy versus Future Cure for Diabetes: What Do We Need?

The β cells contained within the islets of the pancreas are the only cells that exhibit appropriate glucose-responsive insulin secretion. Transplantation of the pancreas, of islets, or of β cells can establish exogenous insulin independence; these three tissue sources are thus far the only known potential cures for diabetes. At the end of October 2002, full pancreatic organ transplantation had been performed with a very high success rate (84% one-year survival of the transplant) in more than 18,000 patients.[3] However, because of the toxic effects of the lifelong immunosuppression drugs needed to prevent tissue rejection, pancreatic transplantation is an accepted therapy largely for patients who have end-stage renal disease and need kidney transplantation (ie, patients who will already be receiving immunosuppressive therapy). A shortage of organs available for transplantation (only about 5000 pancreases are available annually)[4] has further restricted the number of patients who receive pancreatic transplants. The effects of successful whole-organ pancreatic transplantation in patients with advanced type I diabetes have been dramatic, not only halting the progressive complications of the disease but actually reversing some conditions previously thought irreversible, eg, diabetic neuropathy.

Because the islets constitute only 1% of an otherwise healthy pancreas—whose primary function is production of digestive enzymes—replacement of only the nonfunctioning islets is a more ideal therapeutic strategy than whole-organ transplantation. Until recently, islet transplantation had been plagued by poor success rates: Of the 355 adult islet allograft transplants performed from 1990 through 1999, only 11% resulted in insulin independence for more than one year.[5] These low success rates are now believed to result from two commonly used immunosuppressive medications: cyclosporin and steroid agents (which are known to be deleterious to β cells).

The results of two recent clinical studies[6,7] dramatically increased the feasibility of islet transplantation as a valid treatment for type I diabetes. By using a new combination of antirejection medications (tacrolimus, serolimus, and daclizumab) that does not include steroids, islets were transplanted into 12 diabetic patients, all of whom continued to have insulin production and 80% of whom achieved insulin independence at follow-up one year after transplantation. These results revolutionized the field of islet transplantation. However, to obtain the number of islets needed for insulin independence, each transplant requires two or three donor pancreases. Although problems with immunosuppression and graft survival have

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been alleviated, donor organs for transplantation remain scarce.

Other possible solutions to the problem of donor organ shortage include use of growth factors and extracellular matrix components to expand adult β cells; use of putative endocrine precursors (eg, ductal cells) from adult pancreases; and use of fetal pancreatic progenitor cells.[4,8] All of these possible solutions, however, have limited growth potential. By contrast, genetically modified β cells containing transduced oncogenes can be expanded indefinitely; however, in addition to the abnormal karyotype that results from use of these genetically modified cells, problems with their stability and functionality remain a problem.[4] An ideal cell replacement for insulin-deficient states would be available in unlimited supply, have a normal karyotype (number and type of component chromosomes), and show normal functionality of the mature β cell.

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Embryonic stem cells fulfill the first two criteria—and possibly the third—if they can be induced to differentiate efficiently into mature β cells and release insulin appropriately in response to glucose.

What is a Stem Cell?

A mammalian stem cell is a primitive cell that is totipotent: If properly stimulated, this cell can develop into any cell type in the body. The stem cell is unique in its capacity for both self-renewal and differentiation; cell division of a stem cell need not produce two replicate cells but instead may produce, for example, one stem cell and a highly differentiated cell. Stem cells may be further classified as adult (present in many adult mammalian tissues) or *embryonic-stage* (derived from early embryos in the blastocyst stage). Blastocysts are stored by in vitro fertilization clinics. In this article, unless otherwise specified, we refer to embryonic-stage (ES) stem cells when we speak of stem cells.

Some controversy has been raised regarding plasticity of adult stem cells,[9] but ES cells have the intrinsic ability to become mesoderm, ectoderm, or endoderm, thus giving rise to every differentiated cell in the body. ES stem cells express the enzyme telomerase that enables the chromosomes to maintain telomere length after cell division. Because they have high telomerase activity (as do tumor cells), stem cells maintain their proliferative potential and theoretically have unlimited expansion in culture; unlike tumor cells, however, stem cells retain a normal karyotype.

How Can Renewable Stem Cells Be Maintained in Culture?

A key characteristic of embryonic-stage stem cells is their fundamental ability either to remain pluripotent or to differentiate; the mechanism of this determination in human stem cells is largely unknown. By contrast, mouse stem cells have been studied for 20 years, and the general evolutionary conservation of biochemical pathways has led to the assumption that methods developed for murine systems may be applicable to human systems as well. Unfortunately, however, this assumption is not accurate. In culture of mouse stem cells, renewal of stem cells is regulated by leukocyte inhibitory factor (LIF): Removal of LIF causes mouse stem cells to differentiate spontaneously. Although this pathway in mouse stem cells has been well described as the accepted "stem cell renewal" pathway,[9] the pathway in human stem cells now appears to be different. Moreover, neither murine nor human LIF maintains human stem cells in the pluripotent state; a still-unidentified controlling factor is involved.[11,12]

Use of fibroblast feeder layers is necessary in human stem cell development, but possible transfer of harmful mouse viruses to any human transplant recipient precludes clinical use of existing human cells in these patients. In Singapore, however, new human stem cell lines have recently been derived without exposure to mouse cells.[13] This development escapes the problem of transmission of mouse viruses and therefore is an important step toward clinical application. Unfortunately, at this time, the only human stem cells that investigators in the US are permitted to use predate the Singapore technique, so all stem cell lines used in this country are compromised for clinical use because of the potential mouse viral load. Thus, in summary, the requirements for self-renewal of human stem cells are unknown, but unknown factors secreted by fibroblast feeder layers are certainly crucial for maintaining pluripotency and self-renewal. Elucidating the molecular mechanisms involved will be necessary for maintenance of self-renewal and to control differentiation into particular cell lineages.

Culture of human stem cells at this stage is both time-consuming and difficult: Human stem cells grow as clusters or as colonies and do not survive well as single cells. Human stem cells have a doubling time of 30 to 40 hours and thus grow slowly. These cells are fastidious about culture conditions and will either die or differentiate if not kept in a highly specific environment in vitro. Major improvement of culture methods will be difficult to accomplish but will be necessary for development of stem cell clinical applica-

tions. As for all cell-based forms of therapy, FDA regulations for clinical use will require all manipulation of human stem cells during drug development to comply with good manufacturing practice and to maintain proper manufacturing conditions for drug development.

How Can We Influence Stem Cells to Differentiate Into Pancreatic Islet β Cells?

Even under the best circumstances of tissue culture, differentiation into various cell types occurs spontaneously (the default pathway is development into neurons). Only 1% to 3% of spontaneously differentiated human stem cells produce insulin; and unlike mature islet β cells, these stem cells have not been shown to secrete the hormone in response to glucose.[14] Working with mouse stem cells, Lumelsky and colleagues[15] were able to increase the number to 30% using an experimental strategy of stepwise selection through changes in culture conditions. By using a gene trap model to select β cell precursors, Soria and coworkers[16] have obtained an insulin-secreting cell clone from undifferentiated mouse stem cells; the clone was subsequently able to secrete insulin in response to glucose in vitro and in vivo. Whether these methods will be successful with human stem cells is not yet known, but one of the most important steps for clinical application will be the ability to generate pure populations of β cells from human ES stem cells.

Separation of β cells from heterogeneous human stem cell populations is possible by flow cytometry based on cell surface molecules; unfortunately, this method of cell separation is unlikely to be practical, because human ES stem cells must be in clusters to survive; they do not survive as single cells. Generation of pure populations of β cells will require a multistep protocol that begins with induction of endoderm, followed by selection of endocrine progenitors and differentiation into mature β cells. This outcome (differentiation) could be achieved by one or more of three complex strategies:

- appropriate use of growth factors and extracellular matrices;
- selection of endocrine precursors using, for example, gene traps;
- forced expression of transcription factors necessary for the β cell lineage; and
- in vivo growth to complete the differentiation process.

Having examined effects of several growth factors on in vitro differentiation of human ES stem cells, Schuldiner and colleagues[17] showed that hepatocyte growth factor and nerve growth factor are the only growth factors that induce endodermal differentiation. Because pancreatic tissue arises from the endoderm, treatment with these two growth factors could initiate the first step toward differentiation into β cells.

After development into endodermal lineage has been induced, targeted antibiotic protection and gene traps may be used to further select endocrine precursors on the basis of promoter activity. This result can be achieved by infecting endodermal cells with viruses containing β cell gene-specific promoters that drive an antibiotic resistance gene (eg, the gene for antibiotic resistance to neomycin). The only cells surviving culture in the presence of the antibiotic would be cells expressing the β cell gene of interest, and this result would thus yield a pure population of β cell precursors. These genes could be one of several transcription factors present during early stages of β cell differentiation.[18] Another possible method for obtaining a more homogeneous population would be to use antibiotic selection to force expression of relevant β cell transcription factors in endodermal cells.

Extensive genomic and proteomic analysis on these transduced lines will be necessary for ensuring that their genetic integrity has not been compromised. After cells containing genes specific for β cells have been selected, further differentiation could be initiated either with growth factors, with matrices known to induce β cell maturation (eg, exendin-4/glp-1, FGF4, nicotinamide, HGF/SF, or activin A/betacellulin), or with both.[4]

Mouse stem cells and rat fetal pancreatic cells share the ability to become fully mature β cells in vitro,[19,20] releasing insulin appropriately in response to presence of glucose. However, previous experience has shown that although β cell markers or insulin production can be induced in vitro in human fetal pancreatic precursors, these cells are glucose insensitive; a special in vivo environment is necessary to achieve glucose-responsive insulin release.[21] The same in vivo signals may be needed for full maturation of β cells derived from human stem cells. Clinical application may require some time after transplantation before the cells become responsive to glucose.

Conclusions

For human stem cell-based therapy to become a reality for patients with diabetes, several important steps must be accomplished:

- Legislation in the United States must be changed to allow generation of new human stem cell

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lines that have not been compromised by co-culture with mouse cells and that offer distinct cell phenotypes to facilitate graft acceptance.

- The molecular mechanisms of cellular self-renewal must be understood more deeply so that we can efficiently maintain human stem cell lines in their pluripotent state. In addition, present culture methods must be improved to generate sufficient cells for clinical use: After stem cells enter the differentiation pathway, their time clock starts and they begin to lose telomerase activity and the capacity to replicate indefinitely. We must therefore learn how to maintain the stem cells in their pluripotent state for clinical use and to induce the differentiation process when needed for transplantation.
- Efficient, safe protocols must be designed for inducing β cell differentiation so that these clinically differentiated cells can normalize blood glucose levels the same way spontaneously differentiated β cells normalize blood glucose levels.

When these questions are resolved, large-scale prevention and reversal of the consequences and complications of type I diabetes mellitus should be made possible through islet cell transplantation. Despite the great political and scientific effort needed to achieve prevention and reversal of type I diabetes mellitus, pilot studies have shown the feasibility of reaching these goals. ❖

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